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INTERACTION OF FLUORESCENCE QUENCHERS WITH THE *n*-(9-ANTHROYLOXY) FATTY ACID MEMBRANE PROBES

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The fluorescence quenching of the *n*-(9-anthroyloxy) (AO) fatty acid probes has been investigated in aqueous dispersions, vesicles of egg phosphatidylcholine and vesicles formed from red cell ghosts. Negatively charged (KI), neutral (acrylamide) and positively charged ($CuSO_4$) quenchers were used to monitor the location of the probes. The fluorescence of the probes, with the exception of the shortest chain (11-(9-anthroyloxy)undecanoic acid) is not quenched by acrylamide when associated with vesicles. This indicates that in association with vesicles, the 9-anthroyloxy moiety of the long chain probes is buried within the hydrocarbon region and thus well shielded from the aqueous phase. Measurements with KI indicate that the probes are present in the membrane at depths corresponding to the position of the 9-anthroyloxy moiety on the fatty acid, and that the quencher itself forms a concentration gradient within the membrane. Very little or no $CuSO_4$ quenching was observed for *n*-(9-anthroyloxy)stearic acid probes (*n*-AS)with $n > 2$, suggesting that in these vesicles Cu^{2+} does not significantly penetrate the bilayer.

Introduction

Amphipathic molecules, exogenously added to membranes, have long been used to study membrane structure and function. Fluorescent probes, because of their exceptional environmental sensitivity, have been employed to monitor the micro environment of membranes. Ideally, the probe's fluorescence reflects the nature of its immediate environment, and appropriate measurements allow information to be inferred about the local membrane structure. There are, however, several aspects of this approach which complicate the interpretation of the fluorescence in terms of membrane structure [1–3]. The issue that is of primary concern in this paper is the location of the probe relative to the membrane architecture. Interest in this issue has been made particularly acute by the recent work of Conrad and Singer [4], that calls into question the assumption that amphipathic probes are located within the hydrophobic region of biological membranes. Although direct methods such as X-ray diffraction and NMR have been employed to determine the probe disposition in lipid bilayers [5,6], these methods have not been applied to biological membranes. Recently, however, energy transfer to hemoglobin has been used to locate the series of *n*-(9-anthroyloxy) fatty acid (*n*-AO) probes in intact red cell membranes [7]. Since it is difficult to trap the high concentrations of hemoglobin necessary for such measurements in other membrane systems, it is of interest to develop a method of locating these probes which is easier to apply to membranes in general.

In this study we have made use of fluorescence quenching by KI (negative), acrylamide (neutral) and $CuSO_4$ (positive) to obtain information on the

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location of the *n*-AO fatty acid probes in vesicles prepared from egg phosphatidylcholine and red cell ghosts. This approach was first used by Thulborn and Sawyer [8] who investigated the quenching by Cu^{2+} of some of the *n*-AO probes in vesicles of dimyristoylphosphatidylcholine. It is implicit in such studies that the location of the quencher is known and its mode of action is reasonably well defined. On closer examination, however, many of the issues that are raised in connection with the use of fluorescence probes can also be raised in connection with quenchers themselves. It is unclear, for example, how deeply into the bilayer water-soluble quenchers can penetrate. It is also, in general, unclear by what mechanism quenching occurs and, since relatively high quencher concentrations are used, what effect they may have on membrane structure. In the present study we have attempted to resolve some of these issues by comparing the mode of interaction between fluorescence probes and several quenchers in increasingly complex systems. We have also used these results to infer information about membrane structural features that vary with depth in the bilayer.

In agreement with other studies [5–9], we find that most of the *n*-AO probes partition into lipid bilayers and membranes so that their anthroyloxy moieties are positioned at increasing depths in the bilayer. This was inferred from the lack of acrylamide quenching of the probes in the presence of membranes and from the quenching with KI which varies inversely with the acyl chain position of the AO moiety. The mechanism of quenching by acrylamide and KI appears to be a collisional one, while the action of Cu^{2+} is more complex. The fluorescence quenching results and lipid/water partition studies of KI suggest that some form of molecular iodide creates a concentration gradient as a function of depth into the bilayer. Studies of the effect of KI on the leakage of 5(6)-carboxyfluorescein trapped within the internal aqueous compartment of lipid vesicles and the lack of influence of KI on acrylamide quenching of AO probes in membranes, suggest that KI does not significantly alter the membrane structure at the concentrations used in this study.

Materials and Methods

The fluorescent probes 2,3,6,7,9,10,12-(9-anthroyloxy)stearic acid (2,3,6,7,9,10,12-AS), 11-(9-anthroyloxy)undecanoic acid (11-AU), and 16-(9-anthroyloxy)palmitic acid (16-AP) were obtained from Molecular Probes (Plano, TX). Anthracene-9-carboxylic acid (A9C) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Absorption and fluorescence spectral measurements and thin-layer chromatography were used to assess the purity of the fluorophores. Tris-NaCl buffer (20 mM Tris/50 mM NaCl, pH 7.4 at room temperature) was used as the aqueous solvent in all experiments. 8 M acrylamide (Sigma Chemicals or Bio-Rad Laboratories), 4 M potassium iodide (Fisher) and 2 M $CuSO_4$ (Fisher) stocks were prepared in water (for KI, 10 mM $Na_2S_2O_3$, was added to reduce the formation of I_2, I_3^-, etc.). Egg phosphatidylcholine was obtained from Avanti Polar Lipids Inc. and used with further purification.

Vesicles. Small unilamellar egg phosphatidylcholine vesicles were prepared by sonication of lipid dispersions using the method of Huang and Thompson [10]. Lipid that had been stored at −20°C in chloroform was dried in a rotary evaporator, evacuated overnight and suspended in Tris buffer at a concentration of 15 mM phospholipid. This suspension was sonicated for 1 h at 4°C under nitrogen using a Branson sonicator set at approx. 70 watts. Titanium particles released by the sonication tip were removed by centrifugation in a Sorvall SS-34 rotor at 16000 rpm for 35 min and the vesicles were stored under nitrogen at 4°C. These vesicles are a mixture of small unilamellar vesicles and a small quantity (< 5%) of larger multilamellar liposomes [10]. Lipid concentration was determined as inorganic phosphate by the method of Gomori [11]. Band 3 membrane vesicles were prepared from fresh human red cells essentially by the method of Wolosin et al. [12] and were stored at −20°C until thawed and diluted in Tris buffer.

Probe incorporation. To incorporate the fluorescent probes into solutions containing band 3 and phosphatidylcholine vesicles, concentrated stock solutions of probe in ethanol were added while vortex mixing an approx. 1 mM phospholi-

pid suspension of vesicles. The concentration of ethanol in the same volume was usually 0.2%, but occasionally as high as 2%; separate experiments demonstrated that the fluorescence lifetime of AO probes in vesicles was unchanged for 0–10% (by volume) amounts of ethanol. Since the probes exhibit very low quantum efficiencies in water, the time-course of their uptake into vesicles can be followed by steady-state fluorescence, equilibrium was reached within 30 min. For fluorescence lifetime and intensity measurements, the lipid-to-probe ratio in band 3 vesicles was 70 : 1 and in phosphatidylcholine vesicles about 100 : 1. The aqueous dispersions of the probes were formed by vortex mixing a small aliquot (< 0.2% by volume) of the ethanolic probe stock into buffer to give a final probe concentration of about 20 μM. At this concentration the fluorescence intensity was sufficient to perform the quenching measurements and we have assumed that these dispersions result in the formation of micelles.

Fluorescence measurements. All fluorescence measurements were carried out in 10-mm pathlength cuvettes containing 2.5 ml of solution at room temperature. Appropriate aliquots of quencher were added directly to the cuvette and then mixed using a Pasteur pipette. Inner-filter effects due to the quenchers were negligible at the probe excitation (383 nm) and emission wavelengths (400–500 nm). Fluorescence intensity measurements were made using a Perkin-Elmer MPF-2A fluorescence spectrophotometer with excitation and emission slits set to 4 nm. All probes were excited at a wavelength of 383 nm and their fluorescence was scanned from 400 nm to 480 nm. Relative intensities were measured at fluorescence peaks and the data was adjusted for vesicle scattering and dilution due to the addition of quencher.

Fluorescence lifetimes were determined by the phase-modulation technique in an instrument built by SLM Instruments [13]. Measurements were performed with an excitation monochrometer set at 383 nm, a Corning 3144 emission filter (high-pass, cutoff at 400 nm) and an excitation modulation frequency of 18 MHz. These wide band filters effectively average the decay across the emission band. Thus the observed decay is approximately mono-exponential and at 18 MHz the phase and modulation lifetimes are nearly identical. The values used in the present study are, therefore, the average of the modulation and phase values. Uncertainties were determined by estimating the standard deviations from repeated measurements. An excitation polarizer set at 35° from the vertical was used in all lifetime measurements in order to eliminate the effects of Brownian rotations [13]. Measurements were also made using a blank containing all components except the probe, and when necessary, appropriate corrections for scattering contributions were applied to the lifetimes [14].

Analysis of fluorescence quenching. Quenching of fluorescence can occur by several mechanisms [15]. In the case of the collisional (dynamic) quenching both lifetime and intensity are quenched to the same degree by a diffusive process whose kinetics are given by the Stern-Volmer relationship,

$$\tau_o/\tau = I_o/I = 1 + K_{SV}[Q] = 1 + \tau_o k_q[Q] \qquad (1)$$

in which τ_o and I_o are the lifetimes and fluorescence intensity in the absence of quencher and τ and I, the respective values in the presence of quencher of molar concentration [Q]. K_{SV} is the Stern-Volmer quenching constant in M^{-1}, and k_q is the quenching rate in $M^{-1} \cdot s^{-1}$. To a first approximation, k_q is given by [15]

$$k_q = \frac{N}{1000} p(R_F + R_Q)(D_F + D_Q) \qquad (2)$$

in which N is Avogadro's number, p is the quenching probability, R_F and R_Q are the radii and D_F and D_Q the diffusion coefficients of the fluorophore and quencher, respectively. Quenching of the fluorophore's excited state may also occur by a static process which results in an exponential increase in I_o/I with quencher concentration. In the present study, however, no significant exponential dependence was observed and we have therefore analyzed the quenching of the excited state in terms of the Stern-Volmer kinetics of Eqn. 1. In addition to processes involving the excited state, the fluorescence intensity may be quenched by the formation of ground-state complexes. Since this involves the association of the quencher with the fluorophore in its ground state to form a nonfluorescent complex, the kinetics will exhibit the same concentration dependence as dynamic quenching (K_{SV} of Eqn. 1 is replaced by an association

constant K_a). These two processes may be distinguished, however, since the formation of ground state complexes does not affect the lifetime.

To analyze the KI results, in which the quencher appears to form a concentration gradient within the membrane, it is necessary to relate the quencher concentration at the locus of the fluorophore [Q] to the total quencher concentration added to the vesicle dispersion [Q_T]. If the association of the quencher with the membrane can be described by a partition then

$$[Q] = K_P[Q_T] \tag{3}$$

in which K_P is the partition coefficient for Q into a slab at a particular depth within the bilayer. An alternative description of this association in terms of a saturable binding, involving a finite number of binding sites and an association constant, leads to a downward curvature of an I_o/I versus [Q_T] plot [8]. In our study, however, the KI quenching plot was linear, suggesting that partitioning is the most appropriate description of the association of quencher with the membrane.

Average iodide partition coefficient. The partition of iodide into phosphatidylcholine vesicles was measured to determine $\overline{K_P}$, the value of K_P averaged over bilayer depth. The amount of iodide associated with the vesicles was determined using the hygroscopic desorption filtration method of Conrad and Singer [4]. Three filters were mounted in a (Sartorius SM 16-3-16) filtration unit. The top filter, which served to retain vesicles, was a polycarbonate film 5 micrometers thick (Nucleopore Corp. 113602) with a nominal density of $6 \cdot 10^8$ pores/cm^2, treated according to the method of Conrad and Singer [4]. The middle filter was an inert glass fiber spacer of 100 μm thickness (Scheicher and Schuell, grade 30 glass) whose function was to keep the underside of the top filter from being wetted by the contents of the bottom filter. The bottom filter was a pad of absorbent white cellulose of 1.0–1.5 mm thickness (Millipore, AP 1003700).

Samples consisting of phosphatidylcholine in Tris buffer and 600 mM KI, 10 mM $Na_2S_2O_3$, plus a trace amount of $Na^{125}I$ or the same solutions without the vesicles, were equilibrated at room temperature for one hour. The samples were then layered on the top filter and after about 10–15 min under moderate vacuum were completely desorbed by the filter. The amount of radioactivity remaining on the top filter C_s (with vesicles), C_B (without vesicles) was determined by gamma scintillation and adjusted for variations in the total amount of added radioactivity. The partition coefficient was calculated according to,

$$K_P = \frac{(C_S - C_B)}{C_A V_L} V_A \tag{4}$$

in which C_A is the radioactivity of the aqueous phase (approximated as the total sample activity since C_s or $C_B \ll C_A$) and V_A is the aqueous volume (also approximated as the total volume since $V_L \ll V_A$). The volume of the lipid phase (V_L) ($8.3 \cdot 10^{-4}$ ml/mM phospholipid) was calculated assuming a vesicle outer radius of 100 Å, inner radius of 60 Å and an area/phospholipid of 70 Å^2 [10].

Results

Acrylamide

Aqueous dispersions of *n*-AO probes were investigated in order to demonstrate that acrylamide is an effective quencher of the anthroyloxy moiety. As shown in Fig. 1, acrylamide is an effective quencher only of 2-AS and 11-AU in these disper-

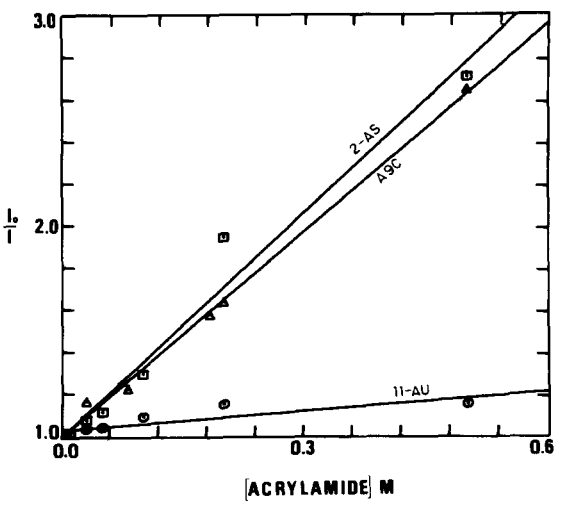

Fig. 1. Fluorescence intensity quenching of AO probes in micelles by acrylamide. Micelles were formed by dispersing 20 μM probe in buffer. Solid lines are the result of least-squares fit to Eqn. 1 and the K_{SV} values (M^{-1}) are 3.5, 3.3 and 0.34 for 2-AS (□), A9C (△) and 11-AU (○), respectively.

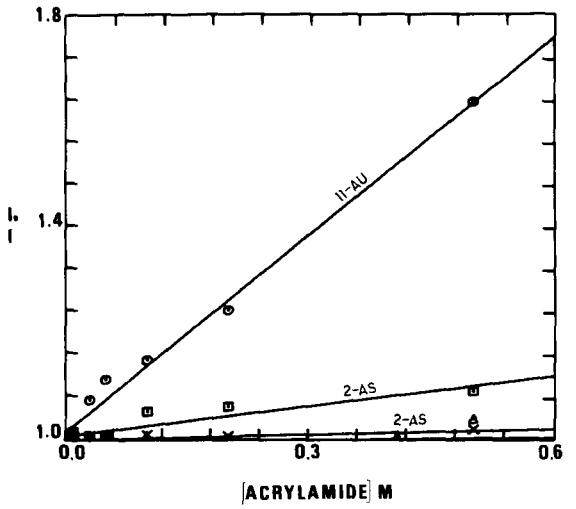

Fig. 2. Fluorescence intensity quenching of AO probes in membranes, by acrylamide. Symbols are: 11-AU (○) and 2-AS (□) both in band 3 vesicles, and 11-AU (△) and 2-AS (×) in egg phosphatidylcholine vesicles. Solid lines are the result of least-squares fit to Eqn. 1. The K_{SV} values are consistent with zero in egg phosphatidylcholine vesicles and 1.5 and 0.2 (M^{-1}) for 11-AU and 2-AS, respectively, in band 3 vesicles.

sions, suggesting that of the long chain probes only the 2-AS has any significant exposure to water. The behavior of 11-AU is generally anomalous relative to the other AO probes in egg phosphatidylcholine and band 3 vesicles, and in water probably reflects a different micelle structure. To confirm that the acrylamide quenching of 2-AS and 11-AU was not peculiar to these probes but reflects the general ability of acrylamide to quench the anthroyloxy moiety, we also investigated the acrylamide quenching of A9C. As seen in Fig. 1, A9C, a water-soluble fluorophore structurally similar to the AO moiety, is efficiently quenched by acrylamide.

Although acrylamide is an effective quencher of *n*-AO in aqueous dispersions, virtually no acrylamide quenching occurs for most of the probes in the presence of egg phosphatidylcholine or band 3 vesicles. The one exception, as seen in Fig. 2, is 11-AU, which is effectively quenched in band 3 but not in egg phosphatidylcholine vesicles. This suggests that unlike the longer chain probes, 11-AU may not partition inside the band 3 vesicle membrane, although it must be associated with some hydrophobic region of the surface since the fluorescence intensity of 11-AU is enhanced (30-fold) in the presence of the membranes.

Potassium iodide

The effect of KI on the fluorescence of aqueous dispersions of AO is shown in Fig. 3. Although all probes exhibit a significant amount of quenching, the behavior is not a simple function of AO acyl chain position. It is likely that the greater rate observed for 2-AS is due to greater exposure to the aqueous phase. The high K_{SV} values of 11-AU and 16-AP may reflect a reduced probability of micelle formation and therefore greater exposure for these shorter chain probes. In contrast, the very small (almost negligible) quenching observed for 3-AS and 6-AS may reflect a tighter packing of these probes in forming micelles. In this regard it is interesting that the crystalline form of the probes exhibit different properties (e.g. color and solubility in ethanol) and, in particular, 3-AS is the most difficult to dissolve in ethanol.

Unlike the complex behavior observed in micelles, the KI intensity quenching in egg phos-

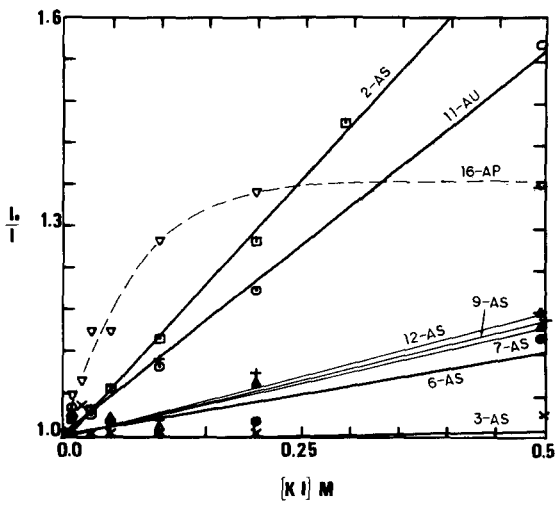

Fig. 3. Fluorescence intensity quenching of AO probes in micelles by KI. Probe concentration was 20 μM. The symbol key is: 2-AS (□), 3-AS (×), 6-AS (●), 7-AS (▲), 9-AS (+) 11-AU (○), 12-AS (△), 16-AP (▽). With the exception of 16-AP, the lines through the data points represent least-squares fits using Eqn. 1 and the corresponding K_{SV} values are listed in Table I. The 16-AP results exhibit considerable deviation from Stern-Volmer behavior and the dashed line was drawn simply to indicate the trend of the data. No explanation is given for this curvature.

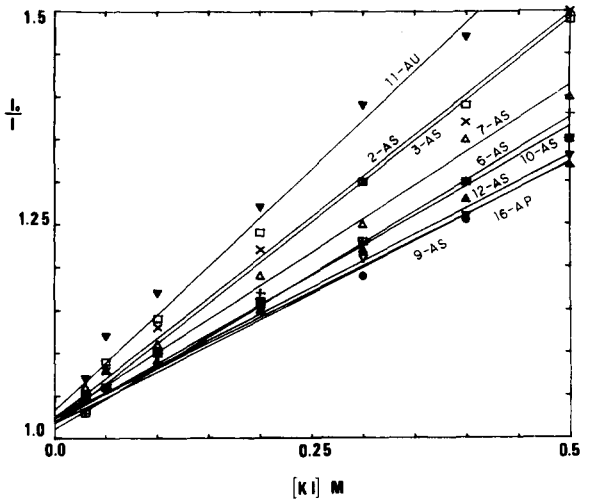

Fig. 4. Fluorescence intensity quenching of AO probes in phosphatidylcholine vesicles by KI. The symbol key is: 2-AS (□), 3-AS (×), 6-AS (+), 7-AS (△), 9-AS (▽), 10-AS (■), 11-AU (▼), 12-AS (▲), 16-AP (●). The solid lines are fits with Eqn. 1 and the corresponding K_{SV} values are listed in Table I.

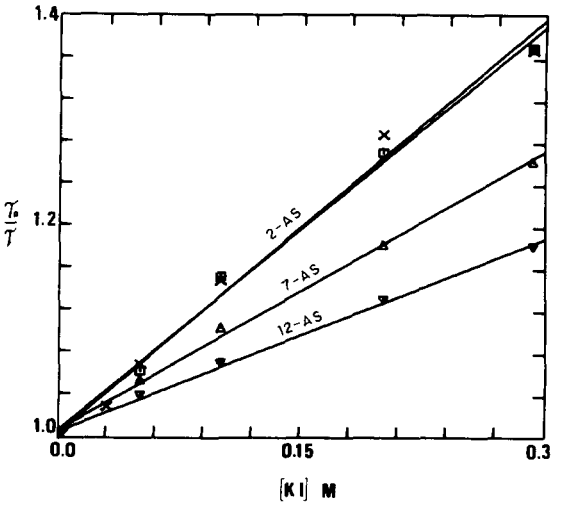

Fig. 6. Fluorescence lifetime quenching of AO probes in membranes by KI. The symbol key is: 2-AS (□), 7-AS (△) and 12-AS (▽) all in band 3 vesicles, and 2-AS (×) in egg phosphatidylcholine vesicles.

phatidylcholine (Fig. 4) and band 3 (Fig. 5) vesicles is linear and exhibits a fairly uniform dependence on n. In addition, as demonstrated in Fig. 6, the quenching of the AO probe lifetimes appears to be linear and about the same magnitude as the intensity quenching. The linearity of the quenching behavior and the agreement between intensity and lifetime quenching suggest that KI quenching occurs by a collisional mechanism. The Stern-Volmer quenching coefficients (K_{SV}) obtained from linear fits to the vesicle data, together with the measured lifetimes and the calculated k_q values are listed in Table I.

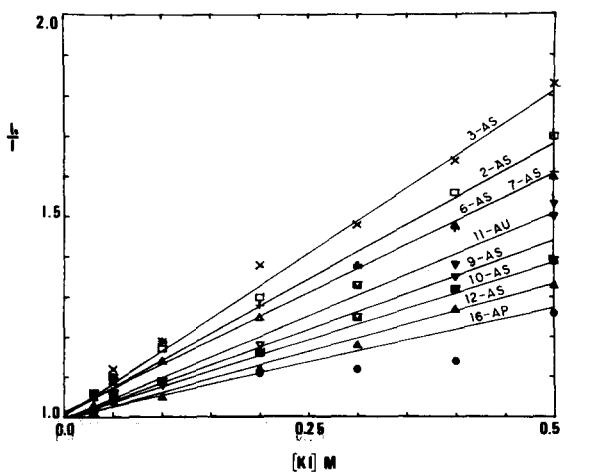

Fig. 5. Fluorescence intensity quenching of AO probes in band 3 vesicles by KI. The symbol key is: 2-AS (□), 3-AS (×), 6-AS (+), 7-AS (△), 9-AS (▽), 10-AS (■), 11-AU (▼), 12-AS (▲), 16-AP (●). The solid lines are fits with Eqn. 1 and the corresponding K_{SV} values are listed in Table I.

Significant quenching by collision will occur only if there is appreciable partition of some form (KI, I^-, I_n^-, etc.) of the quencher into the bilayer. The hygroscopic desorption measurements were performed in order to verify this prediction. As the results in Table II demonstrate, there is an appreciable partition of KI into vesicles, corresponding to $K_P = 6 \pm 4$. Since KI is a chaotropic agent [16] it is possible that KI quenching occurs by an indirect mechanism, in which the perturbation of membrane structure leads to increased exposure of AO probes to water. To examine this possibility, we searched, in egg phosphatidylcholine vesicles, for enhanced acrylamide quenching of 2-AS in the presence of 200 mM KI. After correcting for the reduction in the lifetime (20%) due to the 200 mM KI, no change was observed in the acrylamide quenching rate. To determine if KI could induce leakage, vesicles of egg phosphatidylcholine were prepared in the presence of 20 mM 5(6)-carboxyfluorescein (ultra pure form obtained from Molec-

TABLE I

KI QUENCHING COEFFICIENTS OF THE AO PROBES

K^*_{SV} values were evaluated from lifetime and K_{SV} from intensity quenching. k_q was evaluated according to: $k_q = K_{SV}/\tau_o$ in which τ_o is the lifetime in the absence of quencher. Uncertainties (S.D.) in K_{SV} were estimated from the Stern-Volmer fits to be ± 0.06 M^{-1}. Uncertainties (standard deviations of several measurements) in the lifetime are about ± 0.3 ns. As a result of the compensating weaker fluorescence of the shorter lived probes, these uncertainties for all the probes are about the same.

Medium	Probe	Lifetime (ns)	K^*_{SV} (M^{-1})	K_{SV} (M^{-1})	$k_q(\times 10^{-8})$ ($M^{-1}\cdot s^{-1}$)
Micelle	2-AS			1.40	
	3-AS			0.00	
	6-AS			0.24	
	7-AS			0.32	
	9-AS			0.34	
	11-AU			1.10	
	12-AS			0.36	
	16-AP			**	
Phosphatidylcholine vesicles	2-AS	8.8	1.51	0.93	1.06
	3-AS	10.2		0.93	0.91
	6-AS	10.0		0.73	0.73
	7-AS	10.6		0.77	0.73
	9-AS	10.8		0.61	0.56
	10-AS	11.0		0.69	0.62
	11-AU	10.8		1.12	1.04
	12-AS	12.7		0.61	0.48
	16-AP	12.0		0.59	0.49
Band 3 vesicles	2-AS	8.9	1.36	1.32	1.48
	3-AS	9.9		1.59	1.61
	6-AS	10.2		1.14	1.12
	7-AS	10.3	0.88	1.01	0.98
	9-AS	10.9		1.00	0.92
	10-AS	11.5		0.79	0.69
	11-AU	13.5		1.00	0.74
	12-AS	11.7	0.58	0.65	0.56
	16-AP	12.5		0.41	0.33

ular Probes). Under these conditions the carboxyfluorescein is partially self quenched and does not leak through the vesicles [17,18]. The external carboxyfluorescein was separated from the vesicles by filtration on a Sephadex G-25 column. In the presence of 1% deoxycholate we found that the fluorescence intensity increased by about 400%, indicating that the vesicles were made permeable to carboxyfluorescein. Addition of KI to the intact vesicles partially quenched the fluorescence. However, when 1% deoxycholate was added to the vesicles in the presence of KI, the fluorescence again increased by about 400%. This indicates that even in the presence of up to 500 mM KI the vesicles remain intact and the KI quenching of carboxyfluorescein fluorescence is due to permeation of the quencher through the vesicles.

Copper sulfate

$CuSO_4$ was found to be an effective quencher of all of the AO probes in aqueous dispersions. With the exception of 11-AU and 9-AS, the apparent quenching rate decreases with n. The quenching kinetics were essentially linear and K_{SV} values were in the range $(1-10)\cdot 10^5$ M^{-1}, suggesting that Cu^{2+} binds to these micelles. In both egg phosphatidylcholine and band 3 vesicles the quenching rate (for $[CuSO_4]$ up to 2 mM) was at least two to three orders of magnitude less than observed in aqueous dispersions. Indeed, in egg phosphati-

TABLE II

AVERAGE LIPID/WATER PARTITION COEFFICIENT OF IODIDE

Radioactivity is given in gamma counts per minute of ^{125}I. The egg PC phospholipid concentration was 2.4 mM and the KI concentration was 600 mM. C_A is the aqueous phase activity and C_s and C_B are the filter activities in the presence and absence of vesicles, respectively (see Eqn. 4). $\bar{C}_s$ and $\bar{C}_B$ are the averages of the individual measurements, adjusted for the total amount of radioactivity in the sample before filtration. From these values and Eqn. 4, we find $K_p = 6 \pm 4$. According to the Student's t-test, the probability of $K_p > 1$, is > 0.9.

C_A ($\times 10^{-6}$)	C_s ($\times 10^{-3}$)	$\bar{C}_s$ ($\times 10^{-3}$)
1.01	19.2	18 ± 7
1.12	14.3	
1.16	14.8	
1.16	30.6	
C_A ($\times 10^{-6}$)	C_B ($\times 10^{-3}$)	$\bar{C}_B$ ($\times 10^{-3}$)
1.51	12.1	7 ± 2
0.79	6.0	
1.01	4.7	

dylcholine vesicles only 2-AS and 3-AS exhibited any quenching. In band 3 vesicles most of the probes were quenched by $CuSO_4$; however, the results were not reproducible. The apparent quenching constant varied by a factor of 10 from sample to sample. Since, in some instances, large increases in light scattering occurred, it is likely that the variation in quenching reflects Cu^{2+}-induced alterations in the structure of the membrane.

Discussion

Our results demonstrate that acrylamide, which is extremely water soluble (8 M), is an effective quencher of aqueous dispersions of 2-AS, 11-AU and A9C fluorescence and hence, of the anthroyloxy moiety. In association with vesicles of egg phosphatidylcholine and band 3, however, the quenching of 2-AS (or any of the other long chain (> 16) AO fatty acids) is negligible. We take this as evidence for a buried location of the AO moiety. 11-AU, on the other hand, while inaccessible in egg phosphatidylcholine vesicles, is readily quenched in band 3 vesicles. This suggests that in association with band 3 membranes, the anthroyloxy moiety of 11-AU does not partition into the acyl region. The quenching rate of 11-AU (approx. $1 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) is about 10-fold less than expected for a diffusion limited process. Hence, as is consistent with the observed enhancement of fluorescence intensity, the location of the fluorophore must be partially shielded from the aqueous phase.

While the acrylamide quenching results indicate that the long chain AO probes can partition into membranes, the KI studies yield more specific information about the location of the fluorescent moiety. The KI quenching rates (k_q) of the long-chain probes decrease monotonically with the position of AO in the acyl chain in both egg phosphatidylcholine and band 3 vesicles (Table I and Fig. 7). The difference in the magnitude of the rates in both types of vesicles, as discussed below, may be due to differences in membrane structure. The rates in aqueous dispersions cannot be compared directly with vesicles, since lifetimes are not known and the negative charge of the micelle probably influences the KI quenching rate. Furthermore, as discussed in Results, the variation in

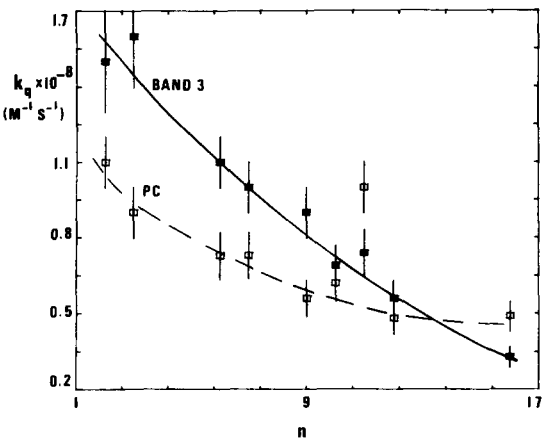

Fig. 7. Rate constants for quenching of n-AO fluorescence by KI in band 3 and egg phosphatidylcholine vesicles. The bimolecular quenching rates (k_q in $M^{-1} \cdot s^{-1}$, from Table I) are plotted as a function of the acyl chain position (n) of the anthroyloxy moiety. Open and closed squares denote values in phosphatidylcholine and band 3 vesicles, respectively. The solid and dashed curves were drawn to emphasize the trend of the data.

quenching coefficient with AO position is probably due to variations in micelle structure.

We interpret the decrease in quenching rate with anthroyloxy position in egg phosphatidylcholine and band 3 vesicles as evidence for the location of the fluorophores at depths corresponding to their position on the acyl chain and for the formation of a quencher concentration gradient in the membrane. The comparison of the results for the two types of vesicles suggests that the relative AO position is similar in both. This conclusion is in good agreement with more direct measurements of the AO location in bilayers and whole red cells. X-ray diffraction studies of oriented bilayers of dipalmitoylphosphatidylcholine containing 12-AS indicate that the anthroyloxy moiety is located near the terminal methyl region of the bilayers [5]. ^{1}H-NMR studies of dipalmitoylphosphatidylcholine vesicles containing 2-AP and 12-AS also suggest a deeply buried location of the 12-AS fluorophore and a more superficial location of 2-AS [6]. In whole red cells, energy transfer measurements have been performed in which hemoglobin (the heme moiety) serves as an acceptor for the AO probes and indicate that the probes occupy positions similar to those in the dipalmitoylphosphatidylcholine bilayers [7,9]. We conclude, in contradiction to Conrad and Singer [4], that fluorescent probes such as the long-chain anthroyloxy fatty acids do partition into biological membranes. On the other hand, some amphipaths (e.g. 11-AU) may adsorb to the surface of the membrane such that their fluorescent moiety does not partition into the acyl chain region.

Our KI study demonstrates that both the intensity and lifetime quenching follow Stern-Volmer kinetics, strongly suggesting that quenching occurs by a diffusion-limited collision mechanism. To estimate whether the observed quenching rates are roughly consistent with those predicted by Eqn. 2, we calculated k_q using: $p = 1$, $R_F + R_Q = 6$ Å and $D_F = D_Q = 3 \cdot 10^{-8}$ cm$^2 \cdot$ s^{-1} (the diffusion coefficient of phospholipids [19]). With these assumptions the k_q value calculated using Eqn. 2 is about $3 \cdot 10^7$ M$^{-1} \cdot$ s^{-1}. The observed value in both egg phosphatidylcholine and band 3 vesicles, using $K_p = 6$, is also about $2 \cdot 10^7$ M$^{-1} \cdot$ s^{-1}. Although this agreement is probably fortuitous, given the broad assumptions concerning various parameters, it does indicate that a diffusion-controlled process is compatible with our results.

Within the framework of a diffusion-limited quenching mechanism the observed gradient is due to the decrease of the product K_pD with depth (assuming the quencher probability p, to be constant). Since D probably increases with depth, the observed quenching gradient must be due to a concentration gradient of the quencher itself, which in turn, may reflect a dielectric gradient. Independent evidence for a dielectric gradient is suggested by the observed increase in anthroyloxy lifetime and quantum yield with acyl chain position, since in isotropic solvents the lifetime is independent of position (Table I and Refs. 8 and 14). Similar behavior has been observed in the variation of the hyperfine splitting constant of spin-labeled nitroxide stearic acids [20].

From Table I and Fig. 7 it is apparent that the KI quenching rate (ignoring 11-AU), for $n \leqslant 12$, is substantially greater in band 3 vesicles than in phosphatidylcholine. Since the diffusion coefficient is probably smaller in band 3 than in egg phosphatidylcholine vesicles, it is likely that the quencher concentration is greater in band 3 vesicles. This is consistent with recent observations indicating that cholesterol reduces the dissolution of water into bilayers [21] with a quencher lipid/water partition greater than 1.

There is one other important difference between KI quenching in the two types of vesicles. As illustrated in Fig. 7, the rate of decrease in quenching rate with acyl chain position is considerably greater in band 3 vesicles and in fact at the $n = 16$ position k_q is smaller in band 3 than in egg phosphatidylcholine vesicles. This difference probably reflects the reduction of acyl chain mobility by cholesterol, the effect of which has been shown to be localized near the center of the bilayer [22,23].

At first sight the 11-AU results appear to be contradictory. If, in band 3 vesicles, the acrylamide quenching of 11-AU is greater than 2-AS (Fig. 2) then how can the order of quenching by KI be reversed? This observation is, in fact, consistent with our finding that the lipid/water partition of the KI quencher is greater than unity. Hence the concentration of the KI quencher at the 2-AS locus is greater than at 11-AU. As seen in

Fig. 7, k_q for 11-AU in phosphatidylcholine vesicles is much greater than expected if its AO moiety were located between the 10 and 12 position. Its k_q value suggests that the fluorophore occupies a position near the 2-AS locus, which would imply that even in egg phosphatidylcholine vesicles the 11-AU fluorophore cannot penetrate deeply into the hydrocarbon region. The alternative explanation, however, that 11-AU has an approximately 2-fold greater diffusion coefficient, cannot be excluded.

Our $CuSO_4$ results are not readily interpreted in terms of probe location. Although efficient quenching is observed in micelles, the rate in vesicles is several orders of magnitude less and does not suggest that $CuSO_4$ (or some form of the Cu quencher) forms a concentration gradient in these vesicles. These results are in apparent contradiction to those obtained by Thulborn and Sawyer [8], who have also investigated the effects of Cu quenching on AO in PC vesicles. In their study, measurements were carried out using vesicles composed of dimyristoylphosphatidylcholine at 20°C, which is below, or in the presence of $CuSO_4$ may span, the phase transition (23.4°C in pure dimyristoylphosphatidylcholine). It is possible that under these conditions, these vesicles may have been much more permeable to Cu^{2+} than the vesicles we use, and therefore a greater degree of $CuSO_4$ quenching would result.

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References

1 Radda, G.K. (1975) in Methods in Membrane Biology (Korn, E.D., ed.), pp. 97–188, Plenum Press, New York
2 Cadenhead, D.A., Kellner, M.J.B., Jacobson, K. and Papahadjopoulos, D. (1977) Biochemistry 16, 5386–5391
3 Ashcroft, R.G., Thulborn, K.R., Smith, J.R., Coster, H.G.L. and Sawyer, W.H. (1980) Biochim. Biophys. Acta 602, 299–308
4 Conrad, M.J. and Singer, S.J. (1981) Biochemistry 20, 808–818
5 Lesslauer, W., Cain, J.E. and Blasie, J.K. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1499–1503
6 Podo, F. and Blasie, J.K. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1032–1036
7 Eisinger, J. and Flores, J. (1982) Biophys. J. 37, 6–7
8 Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 511,125–140
9 Shaklai, N., Yguerabide, J. and Ranney, H.M. (1977) Biochemistry 16, 5585–5592
10 Huang, C. and Thompson, T.E. (1974) in Methods in Enzymology, Vol. 32 (Fleischer, S. and Packer, L. eds.), pp. 485–501, Academic Press, New York
11 Gomori, G. (1942) J. Lab. Clin. Med. 27, 955–960
12 Wolosin, J.M., Ginsburg, H. and Cabantchik, Z.I. (1977) J. Biol. Chem. 253, 2419–2427
13 Spencer, R.D. and Weber, G. (1969) Ann. N.Y. Acad. Sci. 158, 361
14 Matayoshi, E.D. and Kleinfeld, A.M. (1981) Biophys. J. 35, 215–235
15 Birks, J.B. (1970) Photophysics of Aromatic Molecules, John Wiley and Sons Ltd., London
16 Hatefi, C. and Hanstein, W.G. (1969) Proc. Natl. Acad. Sci. U.S.A. 62, 1129–1136
17 Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489–492
18 Ralston, E., Hyelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137
19 Cherry, R. (1979) Biochim. Biophys. Acta 599, 289–327
20 Griffith, O.H., Dehlinger, P.J. and Van, S.P. (1974) J. Membrane Biol. 15, 159–192
21 Simon, A.S., McIntosh, J.J. and Latorre, R. (1982) Science 216, 65–66
22 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265–279
23 Godici, P.E. and Landsberger, F.R. (1975) Biochemistry 14, 3927–3933